

# Production of Ochratoxins in Different Cereal Products by *Aspergillus ochraceus*<sup>1</sup>

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The effects of temperature and length of incubation on ochratoxin A production in various substrates were studied. The optimal temperature for toxin production by *Aspergillus ochraceus* NRRL-3174 was found to be around 28 C. Very low levels of ochratoxin A are produced in corn, rice, and wheat bran at 4 C. The optimal time for ochratoxin A production depends on the substrate, ranging from 7 to 14 days at 28 C. Ochratoxin B and dihydroisocoumaric acid, i.e., one of the hydrolysis products of ochratoxin A, were produced in rice but at levels considerably lower than ochratoxin A. No ochratoxin C was produced in rice at 28 C. When added to rice cereal or oatmeal, the toxin was found to be very stable over prolonged storage and even to autoclaving for 3 hr.

The discovery of toxicity produced in corn by *Aspergillus ochraceus* (10) led to the isolation of three chemically related toxic metabolites, i.e., ochratoxins A, B, and C (6). Ochratoxin A has been shown to be toxic to a variety of laboratory animals (8, 16). Although natural outbreaks of this kind of food intoxication have not been reported, *A. ochraceus* has been readily isolated from many cereal and other food products. The occurrence of this toxin in poor-grade corn (14) and moldy wheat (13) has been demonstrated. In addition to *A. ochraceus*, *A. sulphureus*, *Penicillium viridicatum* (12), and *A. melleus* (M. Lai, G. Semeniuk, and C. W. Hesseltine, *Phytopathology* 58:1056, 1968) also have been shown to produce ochratoxin A. Studies have been published on the detection and estimation of the ochratoxins (1, 4, 11) and also on their microbiological and toxicological properties (2, 5, 8).

Adequate control measures can not be determined until more is known about the conditions under which the toxin is produced. This study was undertaken to determine the effects of incubation time on total ochratoxin A production, the occurrence of other ochratoxins in the substrate, and the effects of various substrates on toxin formation. The stability of ochratoxin A in food stored under different conditions was also studied.

## MATERIALS AND METHODS

**Organism.** The culture used for ochratoxin production throughout this study was *A. ochraceus* NRRL-3174 kindly supplied by C. W. Hesseltine, Northern Regional Research Laboratory, USDA, Peoria, Ill. The culture was grown on potato-dextrose-agar slants at room temperature and stored at 5 C.

**Culture.** The substrate, either 20 or 30 g, was placed in a 500-ml Erlenmeyer flask, soaked with an equal amount of tap water for 2 hr, and autoclaved for 20 min at 121 C. Flasks were inoculated with spores from 1- to 2-week-old slants. The flasks were incubated in the dark and shaken once a day to break the mycelial mass. Five temperatures (4, 15, 20, 28, and 37 C) and four foods (wheat bran, corn meal, polished rice, and bleached flour) were studied. Methods used for extraction, analysis, and preparation of secondary standards have been described previously (1).

**Toxin stability.** To study the stability of the toxin in cereals, 18 µg of purified ochratoxin A in 0.5 ml of sterilized, distilled water was added to 10 g of food. The toxin-containing food was transferred to a plastic bag, flushed twice with nitrogen, and vacuum-sealed. These were stored at 4 and 28 C for various times, and two assays of 5 g each were made for individual samples. To determine the stability of the toxin to autoclaving, 40 µg of ochratoxin A in 0.5 ml of sterilized, distilled water was added to 100 g of cereal in a 500-ml Erlenmeyer flask. Either no water, 50 ml of distilled water, or 50 ml of 20% acetic acid was added to each flask. The samples were autoclaved at 121 C for 0.5, 1, and 3 hr, respectively. Similarly, two analyses of 50 g each were made for individual samples after autoclaving.

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RESULTS

**Effect of temperature and substrate.** Two flasks were harvested for each substrate and temperature at various times over a period of 2 weeks, except in the 4 C experiments which were harvested after 21 and 28 days of incubation. Table 1 shows the results of incubation at 4 C; only slight growth of the fungus was observed at this temperature. The amount of toxin formed is very low; about 3  $\mu$ g of ochratoxin A was produced after 28 days of incubation. This is a contamination level of slightly over 100  $\mu$ g/kg.

Table 2 shows the effect of temperature on ochratoxin A production in polished rice and wheat bran over a 2-week incubation period. Toxin production at 15 C is very slow; however, 1 to 2 mg of toxin is present after 2 weeks. This is a final toxin level of 30 to 60  $\mu$ g/g of substrate. The optimum temperature for ochratoxin A production was found to be 28 C. At this temperature, ochratoxin A was found at levels of 1.5 to 1.8 mg/g of substrate after 7 to 14 days of incubation. Ochratoxin A formation at 37 C was considerably lower than at 28 C.

TABLE 1. Production of ochratoxin A in different cereal products at 4 C

Incubation (day)	Ochratoxin production in					
	Corn		Rice		Wheat bran	
	Micro-grams <sup>a</sup>	Micro-grams/gram	Micro-grams	Micro-grams/gram	Micro-grams	Micro-grams/gram
21	2.67	0.09	0.94	0.03		
28	3.05	0.10	3.11	0.10	2.74	0.09

<sup>a</sup> Values expressed as micrograms of ochratoxin A per 30 g of substrate per flask.

The effects of substrate on ochratoxin A production are shown in Fig. 1. At 28 C, chopped corn was the best substrate for production of ochratoxin A. Polished rice and wheat bran yielded comparable amounts of toxin, but slightly longer incubation was required for maximum production. Bleached flour did not support the production of large amounts of toxin. This may be due to inadequate aeration since the flour formed a paste after sterilization.

The occurrence of ochratoxins other than ochratoxin A was studied in rice incubated at 28 C. Standards of ochratoxin B, ochratoxin C, and one of the hydrolysis products of ochratoxin A, i.e., dihydroisocoumaric acid, were prepared

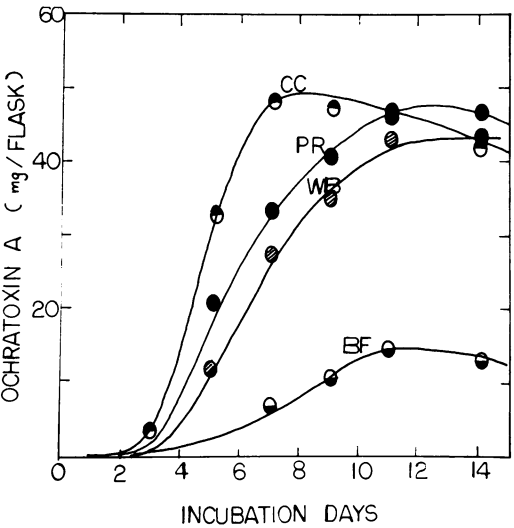


FIG. 1. Production of ochratoxin A in various substrates at 28 C. CC, chopped corn; PR, polished rice; WB, wheat bran; BF, bleached flour; 30 g of substrate per flask.

TABLE 2. Effect of temperature and length of incubation on ochratoxin A production in rice and wheat bran

Incubation (days)	Ochratoxin production at temp <sup>a</sup>							
	15 C		20 C		28 C		37 C	
	PR	WB	PR	WB	PR	WB	PR	WB
3	0.03	0.06	0.01	0.19	0.45	0.37		
5	0.05	0.11	0.10	0.23	29.22	15.79	0.24	0.29
7	0.09	0.12	0.69	8.26	30.23	35.38		
9	0.09	0.10	11.16	11.13	40.48	38.15	15.10	0.38
12	0.54	0.49	14.27	11.08	53.13	44.11	1.33	0.13
14	2.00	1.05	12.09	11.00	39.24	44.39	2.16	0.24

<sup>a</sup> Values expressed as milligrams of ochratoxin A per 30 g of substrate. PR, polished rice; WB, wheat bran.

TABLE 3. *Production of ochratoxin B and dihydroisocoumarin in rice incubated at 28 C<sup>a</sup>*

Incubation (day)	Toxin concn (μg/g)	
	Ochratoxin B	Dihydroisocoumarin
3	4.97	0.86
5	25.59	2.90
7	25.07	3.99
9	44.80	3.33
12	53.59	7.39
14	22.43	6.12
17	26.90	6.58

<sup>a</sup> Incubated with 20 g of substrate per flask.TABLE 4. *Stability of ochratoxin A in cereal products<sup>a</sup>*

Storage conditions <sup>b</sup>	Time (week)	Ochratoxin in			
		Oatmeal		Rice cereal	
		Amt (μg)	Per cent <sup>c</sup>	Amt (μg)	Per cent
4-D	1	18.9	105.0	13.8	76.1
	3	12.0	66.6	10.3	57.2
	12	8.5	47.4	8.7	48.1
28-D	1	18.5	102.8	13.5	75.2
	3	11.2	62.2	8.3	46.1
	12	8.1	45.0	8.5	47.4
28-LD	3	16.4	91.1	6.6	36.7
	12	6.4	35.7	7.6	42.2

<sup>a</sup> Ochratoxin A (18 μg) added to the tested food.<sup>b</sup> Numbers indicate Centigrade temperature. D, experiment carried out in the dark; LD, flasks exposed to light during the day.<sup>c</sup> Per cent of ochratoxin A remaining.

for quantitation of these compounds in the rice extracts. The results of this experiment are shown in Table 3. No ochratoxin C was found in any of the extracts. The amount of ochratoxin B was about 25 times less than the amount of ochratoxin A formed (Table 2). The dihydrocoumarin was present in even lower concentrations.

**Stability of ochratoxin A.** Table 4 shows the stability of ochratoxin A in cereal products. The toxin was very stable when stored in darkness at 4 or 28 C for 1 week. After 12 weeks, about 45% of the toxin was still recoverable from these samples. Although pure ochratoxin A is light-sensitive (7), the effects of light on toxin added to the cereal seemed to be slight, with similar levels in samples stored in the dark and those stored in light and dark.

TABLE 5. *Effect of autoclaving on destruction of ochratoxin A in oatmeal and rice cereal*

Heating conditions		Ochratoxin in			
		Oatmeal		Rice cereal	
		Amt (μg)	Per cent <sup>a</sup>	Amt (μg)	Per cent
None	0	37.34	100	37.90	100
	0.5	6.43	17.22	4.27	11.27
	1	6.45	17.27	4.46	11.77
	3	4.67	12.50	5.30	13.98
50 ml	0.5	12.35	33.07	13.22	34.88
	1	10.83	29.00	10.93	28.84
	3	9.71	26.00	11.94	31.50
50 ml of 20% acetic acid	0.5	14.96	40.06	15.63	41.24
	1	11.79	31.57	11.90	31.40
	3	6.59	17.65	9.46	24.96

<sup>a</sup> Per cent of ochratoxin A remaining.

Autoclaving contaminated samples for up to 3 hr failed to destroy completely the toxin (Table 5). It is of interest that the toxin was more readily destroyed in dry cereal than in the presence of water or even 20% acetic acid. Examination of thin-layer chromatography plates from the autoclaved samples revealed new fluorescent spots; however, the toxicity of these substances is not known.

## DISCUSSION

The optimum temperature for ochratoxin A formation by *A. ochraceus* appears to be very similar to that for aflatoxin formation, 28 C. The major difference is that there is considerable ochratoxin A formation at 15 C, whereas only small amounts of aflatoxin are formed at temperatures in the range of 10 to 15 C (3, 15). Very slow formation of ochratoxins appears to be occurring at 4 C. This fact could be of significance in the storage of grains at low temperatures. Although the amount of toxin present after 4 weeks of incubation at 4 C is very low, continuing production might occur over longer periods. The finding that lower yields of ochratoxin A are obtained at 37 C could be due either to rapid degradation of the toxin at this temperature or decreased toxin formation by the organism. Evidence for the former case might be seen in the presence of considerable toxin after 9 days of incubation at 37 C but a rapid decrease after 3 more days of incubation. These optimal and inhibitory temperatures are certainly dependent upon the strain and species of mold used to produce ochratoxins. *Penicillium viridicatum* shows a considerably lower range and optimal temperature profile than

*A. ochraceus* (A. Ciegler, *personal communication*).

The toxin distribution study indicated that ochratoxin A was the primary toxin produced by *A. ochraceus*. Ochratoxin B and dihydroisocoumaric acid are present at considerably lower levels and are also much less toxic than ochratoxin A. Ochratoxin C which is about as toxic as ochratoxin A is apparently not produced in rice by the isolate used. The optimum incubation time for ochratoxin A production was found to be dependent on the type of substrate used, varying from 7 to 14 days. Schindler and Nesheim found that maximum production of ochratoxin A in shredded wheat occurred after 19 to 21 days at 22 C (9). Although the use of shredded wheat may have affected toxin production, the lower incubation temperature (22 C) would slow toxin production, thus requiring a longer incubation period for maximum yield.

The toxin stability studies revealed that ochratoxin A is a very stable compound which can persist in foods even after 3 hr of autoclaving. This would indicate that removal of ochratoxins from food products might be very difficult, and the best protection would be to prevent toxin formation through satisfactory handling procedures.

#### LITERATURE CITED

1. Chu, F. S., and M. E. Butz. 1970. Spectrophotofluorodensitometric measurement of ochratoxin A in cereal products. *J. Ass. Offic. Anal. Chem.* 53:1253-1257.
2. Davis, N. D., J. W. Searcy, and U. L. Diener. 1969. Production of ochratoxin A by *Aspergillus ochraceus* in a semi-synthetic medium. *Appl. Microbiol.* 17:742-744.
3. Diener, U. L., and N. D. Davis. 1969. Aflatoxin formation by *Aspergillus flavus*, p. 13-54. In L. A. Goldblatt (ed.), Aflatoxin—scientific background, control, and implications. Academic Press Inc., New York.
4. Eppley, R. M. 1968. Screening method for zearlenone, aflatoxin, and ochratoxin. *J. Ass. Offic. Anal. Chem.* 51:74-78.
5. Ferreira, N. P. 1967. Recent advances in research on ochratoxin, part 2: microbiological aspects, p. 157-168. In R. I. Mateles and G. N. Wogan (ed.), *Biochemistry of some food-borne microbial toxins*. M. I. T. Press, Cambridge.
6. Merwe, K. J. van der, P. S. Steyn, and L. Fourie. 1965. Mycotoxins. II. The constitution of ochratoxins A, B, and C, metabolites of *Aspergillus ochraceus* Wilh. *J. Chem. Soc.*, p. 7083-7088.
7. Pitout, M. J. 1969. The hydrolysis of ochratoxin A by some proteolytic enzymes. *Biochem. Pharmacol.* 18:485-491.
8. Purchase, I. F. H., and W. Nel. 1967. Recent advances in research on ochratoxin, part I: toxicological aspects, p. 153-156. In R. I. Mateles and G. N. Wogan (ed.), *Biochemistry of some food-borne microbial toxins*. M. I. T. Press, Cambridge.
9. Schindler, A. F., and S. Nesheim. 1970. Effect of moisture and incubation time on ochratoxin A production by an isolate of *Aspergillus ochraceus*. *J. Ass. Offic. Anal. Chem.* 53:89-91.
10. Scott, de B. 1965. Toxigenic fungi isolated from cereal and legume products. *Mycopathol. Mycol. Appl.* 14:213-222.
11. Scott, P. M., and T. B. Hand. 1967. Method for the detection and estimation of ochratoxin A in some cereal products. *J. Ass. Offic. Anal. Chem.* 50:366-370.
12. Scott, P. M., J. W. Lawrence, and W. van Walbeek. 1970. Detection of mycotoxins by thin-layer chromatography: application to screening of fungal extracts. *Appl. Microbiol.* 20:839-842.
13. Scott, P. M., W. van Walbeek, J. Harwig, and D. I. Fennell. 1970. Occurrence of a mycotoxin, ochratoxin A, in wheat and isolation of ochratoxin A and citrinin producing strains of *Penicillium viridicatum*. *Can. J. Plant Sci.* 50:583-585.
14. Shotwell, O. L., C. W. Hesseltine, and M. Goulden. 1969. Note on the natural occurrence of ochratoxin A. *J. Ass. Offic. Anal. Chem.* 52:81-83.
15. Sorenson, W. G., C. W. Hesseltine, and O. L. Shotwell. 1967. Effect of temperature on production of aflatoxin on rice by *Aspergillus flavus*. *Mycopathol. Mycol. Appl.* 33:49-55.
16. Steyn, P. S., and C. W. Holzappel. 1967. The isolation of the methyl and ethyl esters of ochratoxins A and B, metabolites of *Aspergillus ochraceus* Wilh. *J. S. Afr. Chem. Inst.* 20:186-189.